Dissection of minimal sequence requirements for rhoptry membrane targeting in the malaria parasite

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Abstract

Rhoptries are specialized secretory organelles characteristic of single cell organisms belonging to the clade Apicomplexa. These organelles play a key role in the invasion process of host cells by accumulating and subsequently secreting an unknown number of proteins mediating host cell entry. Despite their essential role, little is known about their biogenesis, components and targeting determinants. Here, we report on a conserved apicomplexan protein termed Armadillo Repeats-Only (ARO) protein that we localized to the cytosolic face of Plasmodium falciparum and Toxoplasma gondii rhoptries. We show that the first 20 N-terminal amino acids are sufficient for rhoptry membrane targeting. This protein relies on both -myristoylation and palmitoylation motifs - for membrane attachment. Although these lipid modifications are essential, they are not sufficient to direct ARO to the rhoptry membranes. Mutational analysis revealed additional residues within the first 20 amino acids of ARO that play an important role for rhoptry membrane attachment: the positively charged residues R9 and K14. Interestingly, the exchange of R9 with a [...]
Dissection of Minimal Sequence Requirements for Rhopty Membrane Targeting in the Malaria Parasite

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Rhoptries are specialized secretory organelles characteristic of single cell organisms belonging to the clade Apicomplexa. These organelles play a key role in the invasion process of host cells by accumulating and subsequently secreting an unknown number of proteins mediating host cell entry. Despite their essential role, little is known about their biogenesis, components and targeting determinants. Here, we report on a conserved apicomplexan protein termed Armadillo Repeats-Only (ARO) protein that we localized to the cytosolic face of Plasmodium falciparum and Toxoplasma gondii rhoptries. We show that the first 20 N-terminal amino acids are sufficient for rhoptry membrane targeting. This protein relies on both – myristoylation and palmitoylation motifs – for membrane attachment. Although these lipid modifications are essential, they are not sufficient to direct ARO to the rhoptry membranes. Mutational analysis revealed additional residues within the first 20 amino acids of ARO that play an important role for rhoptry membrane attachment: the positively charged residues R9 and K14. Interestingly, the exchange of R9 with a negative charge entirely abolishes membrane attachment, whereas the exchange of K14 (and to a lesser extent K16) alters only its membrane specificity. Additionally, 17 proteins predicted to be myristoylated and palmitoylated in the first 20 N-terminal amino acids were identified in the genome of the malaria parasite. While most of the corresponding GFP fusion proteins were trafficked to the parasite plasma membrane, two were sorted to the apical organelles. Interestingly, these proteins have a similar motif identified for ARO.

Key words: acylation, apicomplexa, armadillo repeats, malaria, protein trafficking, rhoptry

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The phylogenetic group called Apicomplexa comprises important unicellular pathogens such as Plasmodium, Toxoplasma and Cryptosporidium. Plasmodium spp. causative agent of malaria, infects an estimated 500 million people annually, resulting in about 1 million deaths each year (1). A key process for apicomplexan parasites is the invasion and subsequent multiplication within their host. This complex process (2–4) is mediated by three types of organelles (micronemes, rhoptries and dense granules) (5,6). The secretion of the proteins stored in these organelles is essential for host cell invasion and is tightly orchestrated and regulated (7–10). Rhoptries are located at the apical pole of invasive-stage parasites (e.g. sporozoites and merozoites in Plasmodium spp., or tachyzoites in Toxoplasma sp.). Toward the end of cell division, rhoptries are first detected as small circular organelles formed by fusion of coated vesicles from the Golgi (so-called pre-rhoptries) located close to the cluster of trans-Golgi vesicles (11,12). The pre-rhoptries mature with the formation of ducts toward the schizont surface close to the developing polar rings (12). It has been proposed that the pre-rhoptry is an endosome-related organelle (13,14). All secreted rhoptry proteins possess a signal sequence that allows entry into the secretory pathway.

Although the role of the rhoptries in host cell invasion has been well established and multiple proteins have been localized to these compartments, their biogenesis and membrane biology is still poorly understood (15). Some studies implicate the adaptor protein 1 complex (AP1) in vesicle trafficking to this organelle (16,17). A more recent study showed a central role of the alveolate-specific dynamin-related protein B (DrpB) in the biogenesis of secretory organelles in Toxoplasma gondii (18). The underlying molecular mechanism that enables trans-Golgi derived vesicles to initiate and propagate rhoptry formation and membrane fusion is still unknown. Studies in both Plasmodium and Toxoplasma localized Rab11A at least temporarily to the rhoptries (19,20). Both proteins, AP1 and Rab11A, are cytosolic factors that are apparently temporarily attached to rhoptry membranes.

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Recently, a protein lacking a signal peptide (PFD0720w) was identified and localized to the apical end of free merozoites of Plasmodium falciparum (21). PFD0720w is characterized by two features: C-terminal armadillo repeats and predicted N-terminal acylation motifs. Armadillo domains are 42 amino acids long motifs implicated in protein–protein interactions (22,23) that consist of three helices with two hydrophobic areas (24). PFD0720w has been termed Armadillo Repeats-Only (ARO) protein. The second feature of this protein, acylation, is an important protein modification involving two types of fatty acids, myristate and palmitate (25). Myristoylation is the permanent co-translational addition of myristic acid to an N-terminal glycine via an amide bond. This process is catalyzed by the enzyme N-myristoyl transferase (NMT). Palmitoylation is the reversible post-translational modification where palmitic acid is linked to a variably modified cysteine via thioester bond. This is catalyzed by membrane-associated palmitoyl acyl transferases (PATs) characterized by the presence of a DHHC motif (26). How membrane specificity of dually acylated proteins is conferred in any system is largely unknown.

Results

ARO is an armadillo-domain containing rhoptry protein that is expressed late in blood stages of P. falciparum and is conserved in Apicomplexa

PIARO (PlasmoDB gene accession number PFD0720w) is located on chromosome 4 in the genome of P. falciparum and comprises 2175 bp with a complex 11-exon structure. Its coding region has a length of 826 bp that results in a protein with a predicted molecular weight of 31 kDa. Transcription is strongly upregulated in late blood stage parasites (27). The gene product is predicted to be involved in invasion (PlasmolINT) and is localized in the apical end of merozoites upon ectopic expression as GFP fusion (21).

Analysis of the primary sequence of PIARO reveals two domains with homology to armadillo-like repeats predicted by MotifScan. Three additional stretches show some homology to armadillo domains, and are predicted to fold in a similar way (Figure S1A, Supporting Information). PIARO is one of 20 proteins that contain several of these repeats. Interestingly, PIARO contains no signal peptide (or recessed hydrophobic patch) as opposed to the vast majority of known rhoptry or microneme proteins (28), but comprises myristoylation and palmitoylation motifs at its N-terminus (Figure 1A). While myristoylation is predicted with a medium confidence score (Myr: 0.569829; NMT: 0.277), palmitoylation of the cysteines C4 and C5 shows high confidence (CSS-Palm: 3.545 and 4.281, respectively) using the available prediction tools.

The expression and localization of the endogenous PIARO protein was analyzed using specific antibodies raised against the full-length protein. Western blot analysis using sorbitol-synchronized parasite material harvested at different time points throughout the asexual life cycle revealed that in line with its transcription profile, PFD0720w is exclusively expressed in late stages as an approximately 30 kDa protein (Figure 1B). Localization studies using PIARO-specific antibodies show an apical distribution in schizonts and free merozoites (Figure 1C), resembling the distribution of the GFP fusion (21). Colocalization with the rhoptry bulb marker RALP-1 (28) (Figure 1D) and the microneme marker EBA-181 identify PIARO as a rhoptry protein (Figure 1E).

PIARO is conserved throughout Apicomplexa with orthologs in T. gondii, Cryptosporidium spp., Theileria spp., Babesia bovis, Eimeria and could also be retrieved from the genome of the dinoflagellate Perkinsus marinus using BLAST searches (Figure S1B). The rhoptry localization of the T. gondii homolog TGME49_061440 was confirmed by overexpression of C-terminal TY1 tagged TgARO and subsequent localization in tachyzoites (Figures S2A–E, S3A).

N-terminal acylation motifs are necessary for peripheral ARO rhoptry membrane attachment

Although ARO does not possess a signal peptide for entering the secretory pathway, it is nevertheless targeted exclusively to the rhoptries, most likely by attachment to the cytosolic face of the membrane by N-terminal lipid modifications. To investigate the role of the predicted myristoylation and palmitoylation motifs, we expressed ARO mutants in P. falciparum lacking the predicted sites (AROΔ25, AROΔ25Δ34, AROΔ2 and AROΔ34, respectively). These PIARO mutants were fused to GFP and localized in transgenic parasites. Deletion of the first 20 amino acid residues (removing the entire region containing the acylation motifs), as well as the mutation of either acylation motif or both of them changed the apical GFP distribution to a cytosolic one (Figure 2A–E). These results suggest that ARO is attached to the cytosolic face of the rhoptry membrane using N-terminal acyl moieties. It also reveals that the armadillo repeats alone do not confer membrane association (Figure 2B).

To confirm the topology of ARO, a Proteinase K protection assay was performed. After digitorin permeabilization of the parasite plasma membrane and degradation of all exposed proteins by Proteinase K, those proteins protected by organelle membranes remain. Following this scheme, ARO is sensitive to Proteinase K treatment, whereas rhoptry internal proteins were protected, confirming its attachment to the cytosolic face of the rhoptry membrane (Figure 3). This topology was also evaluated in T. gondii showing the same results (Figure S3B).

Acylated ARO N-terminus is sufficient for rhoptry membrane targeting

After establishing the necessity of both acylation motifs for ARO membrane association and the apparent independence of the armadillo domains for its targeting, we investigated the minimal sequence requirement for
rhoptry localization. We hypothesized that all necessary information for membrane attachment are encoded within the N-terminal residues. To test this, we fused the first 20 amino acid residues of PIARO (20ARO_wt) to GFP and expressed the fusion protein in the parasite. This minimal construct showed rhoptry localization like the full-length protein. Hence, the N-terminal residues of PIARO encode sufficient information for its membrane specificity (Figure 4A). This minimal construct was co-localized with ARO-specific antibodies to confirm correct organelle recruitment (Figure 4B). Similarly, the first 20 residues of TgARO were fused to GFP-TY1 (20TgARO-GFPTY) showing that this minimal construct is primarily targeted to the rhoptries in T. gondii (Figure S2F,G). Additionally, the expression of 20TgARO in P. falciparum results in rhoptry localization like its malaria counterpart (Figure 4C), supporting functional homology in the well-conserved orthologs.

In order to investigate and provide experimental evidence of the predicted palmitoylation of the ARO N-terminus, we used a biotin switch assay modified for P. falciparum (29). The glideosome-associated protein GAP45 that, like ARO, is predicted to be palmitoylated (as well as myristoylated)
Figure 2: N-terminal lipid modification motifs are essential for Pf ARO rhoptry membrane association. Schematic representation PfARO-GFP fusion protein and localization in unfixed parasites (schizonts and merozoites) of (A) wild type, (B) PfARO Δ120 mutant, (C) PfAROΔA3A4 (double acylation) mutant, (D) PfAROΔ2 (myristoylation) mutant and (E) PfAROΔA5A6 (palmitoylation) mutant. Nuclei stained with DAPI (blue). Enlargement of selected areas are marked with white square and referred as Zoom. Scale bar, 1 μm.

was used as control. This assay involves the substitution of acyl groups by biotin and affinity purification of biotinylated proteins. We show that 20AROWt gets biotinylated and is enriched in the fraction that is expected to contain all S-acylated proteins assayed by this method (Figure 4D). Of note, in contrast to GAP45 that is exclusively detected in this fraction, some background binding of 20AROWt to the matrix could be shown also in the untreated, non-biotinylated one. This might be explained by some unspecific binding of the GFP moiety of the fusion protein to the NeutrAvidin matrix.

Figure 3: ARO is attached to the cytosolic face of the rhoptry membrane. Topology of PIARO-GFP using Proteinase K (PK) protection assay on ARO-GFP permeabilized with digitonin (D). PIARO-GFP was detected with anti-GFP antibodies. Antibodies against a luminal rhoptry protein (anti-RhopH3) and against the cytosolic protein GAPDH (anti-GAPDH) were used as controls. First lane: control, untreated (D− PK−), second lane: permeabilization control (D+ PK−), third lane: permeabilized and proteinase K cleaved (D+ PK+).

Requirements within the first 20 amino acids for rhoptry targeting

The rhoptry membrane specificity cannot be solely dependent on acylation motifs given that other P. falciparum proteins reveal a very similar N-terminal sequence and transcription profile, but are localized to other membranes. For instance, this is the case for PF14_0578 (M\textsubscript{G2}XXC\textsubscript{5}CXX) that is localized to the inner membrane complex (IMC), a membranous system, which underlies the plasma membrane (21,30). In order to dissect the dual function of the N-terminal residues of PIARO – membrane attachment and membrane discrimination – an alanine scan was performed. First, the N-terminal portion of 20AROWt was analyzed (residues 1–10, M\textsubscript{G2}N\textsubscript{4}CCAG\textsubscript{8}R\textsubscript{9}D\textsubscript{10}). While the substitution of the asparagines (20AROA3A4) did not interfere with rhoptry membrane targeting (Figure 5A1) the exchange of amino acid residues 8–10 (20AROA8A9A10) resulted in a cytosolic distribution of the fusion protein (Figure 5B1). This change in the localization pattern is supported by solubility assays: The rhoptry-associated 20AROA3A4 can be detected in all three supernatants (soluble, carbonate and Triton X100 fraction) but 20ARO A8A9A10 is exclusively found in the soluble fraction (Figure 5A2,B2). This result points toward a role of one (or all) of amino acids 8–10 in the acylation process of PIARO. Secondly, amino acid residues 11–20 were exchanged by alanine (20AROA11-20). Although this substitution did not interfere with membrane attachment (Figure 5C2), the specific recruitment to the rhoptry membrane was disrupted and the mutant was directed to the parasite plasma membrane (PPM) (Figure 5C1) – hence this part of the N-terminus of the protein appears to be crucial for rhoptry membrane specific recruitment.

Arginine 9 plays an important role for membrane attachment

To further analyze the crucial involvement of amino acids G\textsubscript{8}R\textsubscript{9}D\textsubscript{10}, two point mutants were generated targeting the
Figure 4: The N-terminus of ARO is sufficient for rhoptry association and is palmitoylated. A) Schematic representation of the minimal PfARO construct (20AROwt) encompassing the N-terminal 20 amino acids fused to GFP and its apical localization in unfixed parasites (S, schizonts and M, merozoites). B) Co-localization in fixed parasites using anti-ARO antibodies. Nuclei stained with DAPI (blue). Enlargement of selected areas are marked with white squares and referred as Zoom. Scale bar, 1 μm. C) Schematic representation and localization of the minimal TgARO construct (20TgARO) fused to GFP (green). Amino acids identical to the P. falciparum counterpart are highlighted or marked with asterisks. Expression and localization of this fusion protein in unfixed malaria parasites (S, schizonts and M, merozoites) showed identical apical distribution as 20AROwt. Nuclei stained with DAPI (blue). D) S-acyl Biotin switch experiment using 20AROwt expressing parasites. Proteins were detected on western blot using anti-GFP and anti-GAP45 antibodies as control. 20AROwt as well as GAP45 is present in the harvested parasite material (input) as well as in the two aliquots (loading, −HA +HA) that were incubated with biotinylation reagent with or without hydroxylamine after NEM treatment. After elution from the NeutrAvidin beads, 20AROwt is enriched in the hydroxylamine (+HA) treated sample when compared with the untreated sample (−HA), while GAP45 is nearly exclusively detected in the +HA sample.
Figure 5: Sequence requirements for ARO rhoptry association using alanine substitutions within the first 20 amino acids. Either the asparagines N3 and N4 (A, 20ARO\textsubscript{A3A4}), the glycine, arginine and leucine at position 7–9 (B, 20ARO\textsubscript{A7A8A9}) or the entire second half of the N-terminus were substituted with alanin (C, 20ARO\textsubscript{A11–20}). Mutant 20ARO-GFP fusion proteins were localized in unfixed parasites (A1, B1, C1). While 20ARO\textsubscript{A3A4} is trafficked to the rhoptries (A1), 20ARO\textsubscript{A7A8A9} reveals a cytoplasmic (B1) and 20ARO\textsubscript{A11–20} a plasma membrane (C1) distribution. Nuclei stained with DAPI. Enlargement of selected areas are marked with white squares and referred as zoom. Scale bar, 1 \(\mu\)m. (A2, B2, C2) This change in the localization pattern is supported by solubility assays: The membrane associated proteins 20ARO\textsubscript{A3A4} (A2) and 20ARO\textsubscript{A11–20} (C2) can be detected in all three extraction fractions (soluble: H\textsubscript{2}O/SN, carbonate Carb/SN and membrane Triton X100: Tx100/SN) while 20ARO\textsubscript{A7A8A9} is exclusively in the soluble fraction (B2) (top panels). Antibodies against the cytosolic protein GAPDH were used as control (bottom panels).

charged residues R\textsubscript{9} and D\textsubscript{10} (G\textsubscript{8} was not addressed since it has similar characteristics as alanine). The substitution of the negatively charged aspartic acid (D\textsubscript{10}) with a positive lysine (20ARO\textsubscript{D10K}) had no impact on localization (Figure 6A1). In contrast, the replacement of R\textsubscript{9} by a negatively charged glutamic acid (20ARO\textsubscript{R9E}) completely abolished membrane attachment (Figure 6B1). This is supported by a changed solubility profile (Figure 6A2, B2). Interestingly, exchange of this positive residue by another similarly charged amino acid, lysine, renders the mutant membrane attached (Figure 6C2) but to the PPM (Figure 6C1). This argues not only for an important
role of this positive charge but also implies a role of the arginine side chain for trapping the protein at the rhoptry membranes. To test a positional effect of the arginine, we generated a construct, where G8 and R9 were shuffled (20AROGR9RG, substitution of G8/R9 with R8/G9). Interestingly, this construct was not targeted to the rhoptries but to the PPM, underlining the importance of this residue (Figure S4A).

Membrane specificity is mediated by charged residues within amino acids 11–20
Although rhoptry membrane recruitment depends on a functional myristoylation and palmitoylation motif, including R9 within the first 10 amino acids, this sequence is not sufficient to direct GFP to the rhoptries (Figure 5C, 20AROA11-20). To further dissect additional determinants, we targeted two features in the second part of the 20

Figure 6: Charged amino acid R9 but not D10 plays a key role in membrane recruitment and specificity of ARO. A) Substitution of the negative charged aspartic acid D10 with a positive charged lysine (20AROD10K) do not alter rhoptry localization (A1) or membrane attachment (A2). B) Exchange of the positive charged arginine R9 with a negative charge residue (20AROR9E) results in a cytosolic distribution of the GFP (B1) and completely abolishes membrane association (B2). Nuclei stained with DAPI (blue). C) Exchange of the positive charged arginine R9 with a positive charged lysine (20AROR9K) results in a plasma membrane distribution of the GFP fusion protein (C1–2). Nuclei stained with DAPI (blue). Enlargement of selected areas are marked with white square and referred as Zoom. Scale bar, 1 μm.
amino acids stretch (L11L12YK14NK15LQE19F): a putative helical structure and charged residues. First, leucines in positions 11 and 12, crucial for helix formation, were exchanged with glycines (Figure 7A1). This mutant (20AROG11G12) showed no impaired rhoptry membrane attachment (Figure 7A2) and therefore argues against the involvement of this secondary structural feature in defining membrane specificity. Next, the involvement of additional positively charged residues within this amino acid stretch was analyzed. Initially, K14 and K16 were substituted with glutamates (20AROK14K16). Interestingly, this mutation localizes the protein to the IMC (Figure 7B1), revealing their involvement in the recruitment of this protein to the rhoptry membrane. The IMC is, like the rhoptries, a Golgi derived compartment and consists of flattened vesicles underlying the plasma membrane. It shows a unique dynamic during schizogony including ring-like formations (21,30–32) as depicted by this construct.

This phenotype was further analyzed by a set of single point mutants. 20AROK14E showed IMC localization (Figure 7C1) like the double mutant 20AROK14K16E. Rhoptry localization could be restored by the introduction of a different positively charged residue 20AROK14R (Figure 7D1). The same set of mutants was generated to investigate the role of K16. The point mutant 20AROK16E revealed a mixed phenotype with partial rhoptry and partial PPM association (Figure S5A1, A3, A4). This phenotype remained unchanged by the exchange of K16 with another positively charged residue 20AROK16R (Figure S5B1). This argues for a somehow subsidiary but facilitating function of K16 for rhoptry membrane recruitment. In contrast, mutation of negatively charged glutamate 19 with the positively charged lysine did not alter rhoptry membrane targeting (20AROE19K, Figure S5C1).

Moreover, the substitution of all amino acids within residues 11–20 except K14 and K16 (20AROA11–20K14K16) resulted in a protein that was predominantly targeted to the rhoptries (Figure S6A1, A3 and A4). Finally, we substituted amino acid residues 12–18 with an unrelated sequence (20AROK12NKS15) that comprised, like the ARO wild type, two positive charges but at a different position (K12 and K15). The localization of this mutant to the rhoptries suggested that the relative position of the charges downstream of R9 is not crucial (Figure S6B1). This data was supported by matching solubilities of the corresponding mutant proteins (Figures S5A2, B2 and C2 and S6A2, B2).

**Predicted dual acylation in the first 20 amino acids confers trafficking of GFP to either the apical organelles, IMC or PPM**

We extended our investigations by testing the membrane-specific targeting of an additional 17 unrelated sequences. These sequences were found on PlasmoDB using the protein motif pattern: M1GX0-17C and further testing their predictions of being myristoylated and palmitoylated in the first 20 amino acids (Figure 8). Expression as GFP fusion proteins in late stages and subsequent localization revealed three distinct phenotypes. First, the N-terminus of PF08_0062 and PFL1119c traffics GFP to the apical pole of nascent merozoites, reminiscent of the rhoptry localization of ARO. Second, the first 20 amino acids of PF14_0578 and PF10_0107 results in a phenotype that is highly characteristic for IMC proteins as previously shown (21,30). Third, the majority of sequences (PF0675w, PF08015w, Mal13P1.51, Mal13P1.310, PF11500w, Mal8P1.109, Mal13P1.44, PFL1090w, PF11_0307, PF14_0354, PF08_0064, PF1005w) resulted in GFP that was targeted to the PPM (Figure 9). Only one fusion protein (Mal7P1.300) resulted in cytoplasmic GFP (data not shown). Taken together, the first 20 amino acids of proteins predicted to be dually acylated confers membrane association. At the same time, this N-terminus can also contain sufficient information for membrane discrimination.

**Discussion**

All apicomplexan parasites possess secretory organelles in their apical region that secrete their contents upon invasion. The detailed underlying molecular machinery that controls and coordinates their biogenesis and maturation has yet to be elucidated.

In this study, we characterized in detail the rhoptry targeting of PFD0720w, a novel and conserved rhoptry-associated protein containing two predicted armadillo-domains termed ARO. ARO is attached to the cytosolic face of the rhoptry membrane. This membrane association is mediated by the means of its N-terminal myristoylation and palmitoylation motifs. Both modifications are known to confer membrane attachment for various parasite proteins like the protein kinases PKG (33) and CDPK1 (34) or the glideosome protein GAP45 (35). These motifs are known to be essential for membrane attachment (34,36–38) and are also required for PFARO. It is predicted that this protein undergoes co-translational modification by myristic acid at the glycine at position 2, followed by post-translational palmitoylation of one or both cysteines at positions 4 and 5. Using a biotin switch experiment on 20AROWtv, that expresses the first 20 amino acids of ARO as a GFP fusion, we provide direct evidence that palmitoylation takes place in vivo. Further, to differentiate the function of the individual cysteines C5 and C6, point mutants were expressed and localized within the parasite (Figure S4B,C). Interestingly, while the alanine substitution of C5 does not interfere with rhoptry membrane association the exchange of C6 relocates the fusion protein to the PPM. This not only points toward C6 as the cysteine involved in rhoptry membrane association, but also underlines the importance of the spatial relationship between the acylated cysteine and the arginine at position 9.
Figure 7: ARO rhoptry membrane specificity is defined by positive charges within amino acids 11–20. A) Secondary structure prediction reveals a helical propensity within amino acids 11–20 (A1). Disruption of this structural feature by substitution of the leucines (L11 and L12) by glycine (20AROG11G12) does not interfere with localization of the GFP to the rhoptries (A2) and membrane association (A3). B) The exchange of the positively charged lysines (K14 and K16) by glutamic acid (20AROE14E16) distributes the GFP fusion protein to the membrane of the IMC (B1) but do not alter the solubility profile of the protein (B2). The apical orientated ring structures are indicative for the nascent IMC (B1). C) Mutation of the lysine 14 (20AROK14E) resulted in the same IMC phenotype but (D) the exchange of lysine with the positively charged arginine (20AROK14R) do not alter rhoptry membrane localization. Nuclei stained with DAPI. Enlargement of selected areas are marked with white square and referred as Zoom. Scale bar, 1 μm.
Although these lipid modifications are essential, they are not sufficient to direct PfARO to the rhoptry membranes. Mutational analysis revealed additional residues within the first 20 amino acids of PfARO that play an important role for rhoptry membrane targeting: the positively charged residues R9 and K14. Interestingly, the exchange of the positive charge at position 9 with a negative charge in PfARO abolishes membrane attachment altogether, whereas the exchange of K14 (and to a lesser extent K16) alters its membrane specificity. The essential role of R9 could be explained by either interference with the myristoylation process (essential for PfARO membrane attachment) or by abrogation of any enzyme-mediated palmitoyl transfer. Following this scenario, R9 (in conjunction with the other positive charges) might play a role for mediating interaction with negatively charged membrane phospholipids and therefore provide additional membrane affinity for PAT activity. Since approximately 30% of the total phospholipids in eukaryotic cell membranes contain negatively charged head groups (39), the importance of positive charges for additional membrane affinity is obvious and was shown for lysines within the N-terminal sequences of the Src family members that, together with the myristoyl moiety, promotes membrane binding (40, 41). Therefore, the exchange of the positive charge at position 9 with a negative charge in ARO could lead to inadequate membrane attachment and consequently abrogate interaction with a PAT, assuming that membrane association promoted by the preceding myristoylation is insufficient for protein palmitoylation. In agreement with this, the substitution of R9 with K does not lead to the loss of membrane attachment (Figure 6C). Nevertheless, the exchange modifies the membrane specificity of the fusion protein suggesting a specific role of the arginine side chain for instance as a recognition motif for a rhoptry membrane specific PAT.

The use of additional 17 unrelated protein sequences encoded in the genome of the parasite and selected on basis of their predicted myristoylation and palmitoylation
motifs further defined the requirements for rhoptry membrane association. Interestingly, all apically targeted fusion proteins (Figures 8 and 9) have (i) an arginine downstream from the predicted palmitoylation site that is separated by two amino acids and (ii) at least one positively charged lysine in close vicinity (Figure 8). For ARO, we showed that the arginine in position 9 (three amino acids downstream of the palmitoylated cysteine) and the most proximal lysine (regardless of position) were essential for rhoptry membrane targeting (Figures 6B, 7C and S4A). In the case of the other apically localized N-termini, R7 and K8 for PF08_0062, and R8 and K11 for PFL1119c, might have an iso-functional role for membrane specificity. Sequence requirements for other compartment-specific membrane associations, like IMC or PPM, have to be experimentally validated in future work. From this study, as for proteins in other organisms (42,43), the PPM emerges as the default localization for dually acylated proteins. Although...
the first 20 amino acids of ARO comprise all trafficking information for correct rhoptry targeting, other proteins might depend on additional information. For instance, full length PFL1090w (GAP45) is an IMC protein; meanwhile, the N-terminal fusion alone is trafficked to the PPM.

It was previously suggested that palmitoylated proteins are recruited to their target membranes by substrate-specific PATs within the membrane of the different organelles (43–46). While the co-translational lipid modification, for instance by myristic acid, mediates low membrane affinity that allow the proteins to cycle on and off intracellular membranes – only the modification by palmitate by a specific PAT is responsible for an increased membrane affinity and trapping of the protein to a specific compartment. This may also include a compartment within the secretory pathway with subsequent sorting and vesicle-mediated trafficking to other compartments.

The genome of Plasmodium encodes at least 13 PATs (47) of which four are transcribed late in blood stages (Table S2, Supporting Information). While a rhoptry membrane bound PAT could mediate PfARO specific targeting, other PATs within the plasma membrane or the IMC could trap PfARO mutants. Alternatively, the specific enzyme responsible for ARO wild type palmitoylation could be localized in specialized compartments of the trans-Golgi defining rhoptry-bound vesicles. Of note, the only putative PATs and identify the putative rhoptry, IMC or plasma membrane bound enzymes.

Interestingly, Blast searches show that ARO is not only highly conserved across Apicomplexan parasites but also retrieved homologs in yeast (Vac8, NCBI GenBank NC_001137.3) and the Paramaecium (Nd9, NCBI GenBank CAC12829.1) with 26 and 24% identity, respectively. All three proteins possess a lipid interacting N-terminus and armadillo protein–protein interaction motieties at the C-terminus. Different studies have shown that Vac8p is involved in vacuole inheritance, homotypic fusion, vacuole-nucleus junctions and cytoplasm-to-vacuole targeting (49–54). In turn, Nd9p is needed for the discharge of Paramaecium trophozoits and was suggested to be involved in their fusion with the plasma membrane (25). Yeast cells deleted for Vac8 do not inherit vacuoles from the mother to the daughter cell and manifest a mild to highly fragmented vacuole morphology (55). Although it is tempting to speculate on a similar function of ARO, for instance for rhoptry membrane fusion, complementation assays using PfARO on a vac8 deficient background does not rescue the fragmented vacuolar morphology and does not complement Vac8 function in an in vitro vacuole fusion assay (Figure S7). Gene disruption experiments are consequently required for deciphering the precise function of ARO in apicomplexan parasites.

Material and Methods

Cell culture and transfection of Plasmodium falciparum

P. falciparum (3D7) was cultured in human O+ erythrocytes according to standard procedures using complete RPMI medium (54). For transfection, ring-stage parasites (10%) were electroporated with 100 μg of plasmid DNA resuspended in cytokine as previously described (56). Transfectants were selected using 10 μM WR99210 (57).

Nucleic acids and constructs

PFD0720w was previously cloned as a GFP-fusion and localized in P. falciparum (22). For the generation of a GST-fusion protein, full length PFD0720w was reamplified and cloned using the EcoRI and Xhol sites of pGEX-4T-1 vector (GE Healthcare). PCR amplifications were performed using Phusion DNA polymerase (New England Biolabs). Mutations of PFD0720w and the synthetic chimaeras were produced by PCR using the respective oligonucleotides summarized in the Table S1. All constructs were digested with KpnI and AvrII and cloned into a derivate of pARL1a (58) containing GFP (37) that ensures expression of C-terminally GFP in late stages (59).

PFD0720w orthologs were identified using the blast search tool on NCBI and the BLAST tool in PlasmoDB and ToxoDB. Protein sequences of all orthologs in apicomplexan parasites were aligned using Praline multiple sequence alignment (www.ibi.vu.nl/programs/praline www).

Genes encoding proteins that are predicted to be myristoylated and palmitoylated were identified using the protein motif pattern tool available in PlasmoDB searching for the pattern M 1GX0-17C in the whole P. falciparum genome. Each sequence was run into the myristoylation and palmitoylation prediction softwares: Myristoylator (60), NMT (61) and CSS-Palm (62).

The 20 amino acid N-termini of PF08_0062, PFL1119c, PF14_0354, PF14_0578, PF10_0107, PF08_0675w, PF11_0307, PF14_0354, PF08_0064, PF11_0050w and Mal7P1.300 were generated using overlapping oligonucleotides, summarized in Table S1. Subsequently, the KpnI/AvrII gene fragments were cloned into the same pARL1a vector used for all other transfection experiments. All constructs were sequenced for accuracy.

Recombinant protein expression and antisera

Mouse antisera was raised against PFD0720w-GST fusion protein. The fusion protein was expressed in Esherichia coli BL21-RIL and induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The GST fusion protein was purified by affinity on a high-affinity GST resin (GenScript) according to the manufacturer instructions. Purity was assessed on SDS-PAGE, and concentration was calculated using its absorbance at 280 nm. Mice were immunized according to good laboratory practice using TiterMax® Gold Adjuvant (Sigma).

Western blot analysis

P. falciparum proteins were extracted using 0.03% saponin (Sigma), and pellets were resuspended in adequate amount of PBS and 5x sodium dodecyl sulphate (SDS) loading dye. Proteins were separated on 10% SDS–PAGE and transferred to nitrocellulose membranes (Schleicher & Schuell). Monoclonal mouse anti-GFP (Roche) was diluted 1:1000, rabbit monoclonal mouse anti-PfARO (Sigma) was diluted 1:500.
anti-RALP1-C (28) and mouse anti-PFD0720w were diluted 1:500 in 5% glycerol in PBS with 0.2 mM biotin-BMCC, and kept at 4 °C for 1 h with rotation. Parasite lysates were then chloroform/methanol precipitated and resuspended in solubilization buffer (4% SDS, 50 mM Tris, 5 mM EDTA, pH 7.4) containing 10 mM NEM. Solubilized protein was diluted with lysis buffer containing 1 mM NEM, 0.2% TX100 and protease inhibitor cocktail (Roche), and incubated at 4 °C for 1 h with rotation. Parasite lysates were then chloroform/methanol precipitated (65), and the pellet was resuspended in solubilization buffer (4% SDS, 50 mM Tris, 5 mM EDTA, pH 7.4) containing 10 mM NEM. Solubilized protein was diluted with lysis buffer containing 1 mM NEM, 0.2% TX100 and protease inhibitors, and incubated overnight at 4°C with rotation. After chloroform/methanol precipitation, proteins were resuspended in solubilization buffer and divided into two equal sets; first set was diluted with control buffer (50 mM Tris, 1 mM bixin-BMCC, 0.2% TX100, pH 7.4) and the second with hydroxylamine buffer (0.7% hydroxylamine, 1 mM bixin-BMCC, 0.2% TX100, pH 7.4), and incubated at room temperature for 2 h. After chloroform/methanol precipitation the pellet was resuspended in solubilization buffer, diluted in low-bixin-BMCC buffer (50 mM Tris, 150 mM NaCl, 5 mM EDTA, 0.2 mM bixin-BMCC, 0.2% TX100, pH 7.4) and incubated at room temperature for 2 h with rotation. The sample was then precipitated again by chloroform/methanol precipitations and resuspended in solubilization buffer. The samples were diluted to 0.1% SDS by addition of lysis buffer with 0.2% TX100 and protease inhibitors, and incubated for 30 min at room temperature with rotation. The samples were centrifuged at 20,000 × g to remove any insoluble material, high-capacity NeutAvdin-agarose (Pierce) was added, and incubated at room temperature for 2 h with rotation. Beads were washed four times with lysis buffer with 0.1% SDS and 0.2% TX100. Beads were eluted with 4× SDS sample buffer containing β-mercaptoethanol and boiled at 80°C. Sample was run on SDS–PAGE and blotted on WB for analysis. Control and hydroxylamine sample was run side by side.

Solubility assays

P. falciparum proteins were extracted using 0.03% saponin (Sigma) (if not otherwise indicated, all steps were carried out on ice). After three times washing with PBS, complete protease inhibitor (Roche) was added and parasites were lysed in 100 μL of water and frozen at −80°C. Preparation was thawed and mechanically disrupted passing through a needle and frozen again at −80°C. Extraction was done sequentially: the lysate was thawed and centrifuged at 14,000 rpm for 5 min. The supernatant was removed, centrifuged again for 5 min to remove residual insoluble material and saved as the soluble protein fraction. The pellet after hypotonic lysis was washed once with PBS and resuspended in 100 μL新鲜ily prepared 0.1 mM Na2CO3 and kept on ice for 30 min to extract peripheral membrane proteins. After centrifugation at full speed for 5 min the supernatant was kept and centrifuged again. The pellet was washed once with PBS and extracted for 30 min with 100 μL 1% Triton X100 and centrifuged at full speed for 5 min to get the integral membrane protein fraction in the supernatant. The final pellet was washed once with PBS and resuspended on 100 μL PBS containing the insoluble fraction. Equal amounts of all supernatants were analyzed by immunoblotting with adequate amounts of 5× SDS loading dye. Proteins were detected using anti-GFP antibodies, the cytosolic protein GAPDH used as a control (63).

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1: Alignment of PFD0720w apicomplexan orthologs (related to Figures 1 and 2). A) Secondary structure prediction (www.cmpbio. dundee.ac.uk/www/grad) of PFD0720w indicates multiple helices as depicted by arrows. Although only two armadillo domains are predicted by MotifScan (http://hits.iah-sib.ch/cgi-bin/PSSCAN), five armadillo domains might be present in PFD0720w given three additional sets of triple helices (orange, red and purple arrows). B) PFD0720w homologs were aligned using ClustalW multiple sequence alignment. Armadillo repeats are in boxes following the same color format than (A). Asterisks (*) show fully conserved residues, colons (:) show conserved residues of a group with
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strongly similar properties and periods (I) show conserved residues of a group with weakly similar properties.

Figure S2. ARo in Toxoplasma gondii co-localize with the rhoptries (related to Figures 1 and 4). A) Domain structure of T. gondii ARo (TgARo); light red: armadillo domains, black: TY1 epitope. B) Co-localization studies of TgARo-TY1 with antibodies against the TY1 epitope (green) and the rhoptry bulb marker ROP1 (red). C) ROP7-TY (red). D) the rhoptry neck marker RON4 (red) and (E) the microneme marker MIC3 (red). Nuclei stained with DAPI (blue). F) Schematic representation and localization of the minimal TgARo construct (20TgARo) fused to GFP (green) and (G) co-localized with the rhoptry marker ROP7 (red). Nuclei stained with DAPI (blue). Generation of cell lines and assays described in Appendix S1, Supporting Information.

Figure S3. TgARo is attached to the cytosolic face of the rhoptry membrane (related to Figure 3 and Figure S1). A) Expression of TgARo-TY1 on transgenic parasites and not in RH wild type. B) Topology of TgARo-TY1 using Proteinase K (PK) protection assay on TgARo-TY1 permeabilized with digitonin (D). TgARo-TY1 was detected with anti-TY1 antibodies. Antibodies against a luminal rhoptry protein (anti-ROP1) and against the cytosolic protein profilin (anti-PRF) were used as controls. First lane D−PK−: not treated control. Second lane D+PK+: permeabilization control. And third lane: D+PK−: permeabilized and digested with proteinase K. Assay described in Supplementary material and methods.

Figure S4: Positional effect of arginine 9 and role of the individual cysteines 5 and 6 in rhoptry membrane attachment (related to Figure 6 and Discussion). A) Substitution of G6/R9 with R6/G9 (20ARO6/9) do alter rhoptry localization of the fusion protein to a plasma membrane association. B) The replacement of C5 with an alanin (20ARO/C5A) had no effect on rhoptry membrane localization of the fusion protein. C) Substitution of the C6 with an alanin (20ARO/C6A) targets the protein to the parasite plasma membrane. Enlargement of selected areas are marked with white squares and referred as Zoom. Scale bar, 1 μm.

Figure S5: Contribution of K16 for rhoptry membrane specificity and expandable negative charge E19 (related to Figure 7). A) Substitution of positively charged K16 by glutamic acid (20ARO/C16E) leads to a mixed phenotype with some cells that show mainly apical (33%), some cells show apical and plasma membrane (47%) and some cells show predominately plasma membrane (20%) targeting of the fusion protein with an extended cytosolic pool (A1–2). The mixed phenotypes were quantified based on 30 cells and categorized. Example for each category (A3) and percentage of cells resembling this phenotype (A4) are given. B) The replacement of K16 with another positively charged residue (20ARO/K16R) had no effect on rhoptry membrane localization of the fusion protein. C) Substitution of the negatively charged E19 with the positively charged residue lysine (20ARO/K16L) does not interfere with rhoptry membrane targeting (A1–2). Enlargement of selected areas are marked with white square and referred as Zoom. Scale bar, 1 μm.

Figure S6: A K14/K16 minimal construct and positional effect of positive charges (related to Figure 7). A) The replacement of all but K14 and K16 within the amino acid 11–20 alanine (20ARO1–1–20ARO14;16) leads to a mixed phenotype with some cells that show mainly apical (7%), some apical and plasma membrane (78%) and some predominately plasma membrane (15%) targeting of the fusion protein with an extended cytosolic pool (B1–2). This mutant cannot fully resemble the wild type targeting sequence and results in a mixed phenotypes quantified in C4. B) The shift of the positive charges from K14/16 to K12/15 by the substitution of amino acid 12–18 with an amino acid stretch of an unrelated protein (PF14_0578, 20ARO12–18-0578) does not impair rhoptry membrane localization (C1–2). Enlargement of selected areas are marked with white square and referred as Zoom. Scale bar, 1 μm.

Figure S7: Vac8 complementation in yeast (related to Discussion). A) Complementation analysis by microscopy. Localization of GFP-tagged PfARoVac8N fusion protein in yeast cells. Wild-type Vac8 and PfARoVac8N were co-transformed into yeast and examined by fluorescence microscopy. Bar, 10 μm. B) Number of vacuoles per cell for all constructs. C) In vitro vacuole fusion assay. Vacuoles purified from tester strains BJ3050 (pep5Δ) and DKYEY281 (pxo8Δ) were incubated in the fusion reaction buffer containing ATP+regenerating system for 90 min at 26°C and then developed. Fusion activity was measured as described in Appendix S1, Supporting Information.

Table S1: Primers used in this study. Restriction endonuclease Sites are underlined.

Table S2: Palmitoyl acyl transferases in P. falciparum (related to Discussion). Late transcribed DHHC palmitoyl acyl transferases (PAT) were retrieved from PlasmoDB. PATs with a transcriptional maximum at 42 ± 4 h with fourfold induction are in black, PATs with < fourfold upregulation in grey.

Table S3: Plasmids and yeast strains used in this study.

Appendix S1: Materials and methods.

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References


